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Molecular detection and the phylogenetics of *Wolbachia* in Chinese spiders (Araneae)

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Abstract. Maternally inherited endosymbiotic bacteria *Wolbachia pipientis* have been shown to have wide-ranging effects on the reproduction of their hosts. This study presents the first survey and characterization of *Wolbachia pipientis* that have infected spiders collected from Wuhan, Hubei Province, China. First, we used universal primers of the *wsp* gene (*Wolbachia* Surface Protein, WSP) to examine the infection of *Wolbachia* in spiders. We found that, out of 31 spider species, 7 species were infected with *Wolbachia*. Then we used the specific primers of the A and B *Wolbachia* supergroups for the *wsp* gene to determine if there are super-infections in these infected spiders. Specimens of *Nephila clavata* were infected with strains of both A and B *Wolbachia*, while the others were infected with either strain A or B. Lastly, we aligned the sequences obtained with published ones to establish the phylogenetic relationships among *Wolbachia* found in spiders. The *Wolbachia* in *Larinia argiopiformis* Bösenberg & Strand 1906, *Eriovixia cavaleriei* (Schenkel 1963), *Araneus ventricosus* (L. Koch 1978), and *Pholcus crypticolens* Bösenberg & Strand 1906 belong to the A supergroup and the other three species, *Nephila clavata* (L. Koch 1878), *Oxyopes sertatus* L. Koch 1877 and *Coleosoma octomaculatum* Bösenberg & Strand 1906 belong to the B supergroup.

Keywords: Phylogenetic analysis, *wsp* gene, super-infection

Wolbachia is a common and widespread group of symbiotic bacteria found in the reproductive tissues of arthropods. These bacteria are transmitted through the cytoplasm of eggs and have evolved various mechanisms for manipulating the reproduction of their hosts, including induction of cytoplasmic incompatibility (CI), parthenogenesis, feminization, male-killing, and modification of fecundity or fertility (Werren et al. 2008). *Wolbachia* is thought to be a major factor in the evolution of sex determination, eusociality, and speciation.

At present eight different supergroups (A–H) of *Wolbachia pipientis* have been recognized based on phylogenetic analysis with the *wsp*, *ftsZ*, and 16S rRNA loci (Lo et al. 2002; Rowley et al. 2004; Bordenstein & Rosengaus 2005). The *wsp* gene encodes a surface protein and is the most rapidly evolving of the three. It is therefore more often phylogenetically informative and the most commonly used *wsp* gene for strain differentiation (Zhou et al. 1998; Rowley et al. 2004). *Wolbachia* strains of supergroups A and B are present in arthropods, whereas C and D are so far found only in filarial nematodes. Strain E is known only from springtails (Lo et al. 2002; Czarnetzki & Tebbe 2004); F exists both in arthropods and nematodes, including termites (*Microcerotermes* sp. and *Kalotermes flavicollis*), filarial nematodes (*Mansonella* spp.), a weevil (*Rhinocyllus conicus*) and two cimicids (Lo et al. 2002; Rasgon & Scott 2004; Casiraghi et al. 2005). Subsequently a lineage of *Wolbachia* outside of A–F supergroups was discovered in Australian spiders (Rowley et al. 2004) and was placed in new supergroup G, while another lineage in termites of the genus *Zootermopsis* has been placed in supergroup H (Bordenstein & Rosengaus 2005).

In some species of arthropods, individuals are infected with more than one strain of *Wolbachia*, which is called *Wolbachia* super-infection (Werren 1997; Zhou et al. 1998; Narita et al. 2007). Since most infected species belong to supergroups A and B, Zhou et al. (1998) designed specific primers of the *wsp* gene for these two groups to quickly identify super-infections. Double infections with supergroup A- and B-*Wolbachia* have been found in over 15 species (Zhou et al. 1998). By using a beta-binomial model, Hilgenboecker et al. (2008) estimated that 66% of insects are infected with *Wolbachia*. Most *Wolbachia* research has focused on insect hosts and non-spider arachnids such as ticks and mites (Vala et al. 2004; Ros & Breeuwer 2009). Although spiders (Araneae) are one of the largest taxonomic groups of arthropods, there are only a few reports of *Wolbachia* infections in spiders (Oh et al. 2000; Cordaux et al. 2001; Rowley et al. 2004; Goodacre & Martin 2006; Baldo et al. 2008). Recent work has also identified the presence of three other reproductive parasites in spiders, *Spiroplasma*, *Rickettsia* (Goodacre & Martin 2006) and *Cardinium* (Duron et al. 2008; Martin & Goodacre 2009). These symbionts have effects on the reproduction of their arthropod hosts very similar to those of *Wolbachia*. Furthermore, Goodacre et al. (2009) reported that *Rickettsia* could modify the long-distance dispersal capacity in a spider host, *Erigone atra* Blackwall 1833 .

Here we tested a range of Chinese spiders for the presence of *Wolbachia* based on the *wsp* gene to determine 1) the occurrence of multiple infections and 2) the phylogenetic relationships of the *Wolbachia* present in Chinese spiders.

METHODS

Spider collection and DNA extraction.—A total of 930 spiders of 31 species and 11 families was collected from the suburbs in Wuhan, Hubei Province, China from March 2006 to April 2008 (see Table 1). Thirty individuals were sampled from each of these 31 spider species for DNA extraction.

Depending on the size of the spider, we harvested 1–4 legs from each individual for DNA extraction. We used leg tissue samples to avoid potentially contaminating gut contents, but also extracted DNA from tissue taken from the abdomens of a subset of specimens. Legs were rinsed with 70% ethanol and then homogenized in Holmes-Bonner buffer. We extracted DNA from the homogenate following standard procedures (Sambrook et al. 1989). DNA extractions of *Wolbachia*-

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Table 1.—Results of PCR-screening for *Wolbachia* using specific *wsp* primers in spiders sampled from Wuhan, Hubei Province, China. All spiders were adults, and 30 individuals for each species of spiders were tested for the rate of *Wolbachia* infection. N.A. indicates no infection found.

Family	Spider species	Rate of *Wolbachia* infection	*Wolbachia* strain
Araneidae	*Larinioides cornuta* Grasshoff 1983	-	N.A.
	Larinia argiopiformis Bösenberg & Strand 1906	40%	*w*Lararg
	Argiope amoena (L. Koch 1878)	-	N.A.
	Eriovixia cavaleriei (Schenkel 1963)	26.7%	*w*Ericav
	Araneus ventricosus (L. Koch 1878)	20%	*w*Araven
	Araneus nitificus (Simon 1886)	-	N.A.
Clubionidae	*Clubiona japonicola* Bösenberg & Strand 1906	-	N.A.
Nephilidae	*Nephila clavata* (L. Koch 1878)	63.3%	*w*Nepcla
Lycosidae	*Pardosa laura* Karsch 1879	-	N.A.
	Pardosa astrigera L. Koch 1878	-	N.A.
	Pardosa pseudoannulata Bösenberg & Strand 1906	-	N.A.
	Pirata piraticus (Clerck 1757)	-	N.A.
	Pirata piratoides (Bosenberg & Strand 1906)	-	N.A.
	Pirata subpiraticus (Bosenberg & Strand 1906)	-	N.A.
	Pirata tenuisetaceus Liu 1987	-	N.A.
Linyphiidae	*Hylyphantes graminicola* (Sundevall 1830)	-	N.A.
	Neriene radiata (Walckenaer 1842)	-	N.A.
	Erigone prominens Bösenberg & Strand 1906	-	N.A.
	Neriene japonica (Oi 1960)	-	N.A.
	Neriene limbatinella (Bösenberg & Strand 1906)	-	N.A.
	Ummeliata insecticeps (Bösenberg & Strand 1906)	-	N.A.
Oxyopidae	*Oxyopes sertatus* L. Koch 1877	73.3%	*w*Oxyser
Pholcidae	*Pholcus crypticolens* Bösenberg & Strand 1906	56.6%	*w*Phocry
Salticidae	*Marpissa magister* (Karsch 1879)	-	N.A.
	Plexippus paykulli (Audouin 1826)	-	N.A.
Thomisidae	*Misumenops tricuspidatus* (Fabricius 1775)	-	N.A.
	Thomisus labefactus Karsch 1881	-	N.A.
Tetragnathidae	*Tetragnatha squamata* Karsch 1879	-	N.A.
	Tetragnatha praedonia L. Koch 1878	-	N.A.
Theridiidae	*Coleosoma octomaculatum* Bösenberg & Strand 1906	13.3%	*w*Coloct
	Achaearanea tepidariorum (C.L. Koch 1841)	-	N.A.

infected *Drosophila melanogaster* served as a template for positive controls in PCR reactions.

PCR detection of *Wolbachia*.—Since the primers hedin-O and hedin-C work well in a range of Araneae, we used these to amplify the D2–D3 region of the spider 28S nuclear ribosomal RNA gene to control for the quality of DNA extracted from each specimen (Goodacre & Martin, 2006). To detect the presence of *Wolbachia*, we performed PCR for the *wsp* gene using the primers 81F and 691R (Braig et al. 1998). The primers for detecting super-infection in spiders are 136F/ 691R (primers for supergroup A *Wolbachia*) and 81F/552R (primers for supergroup B *Wolbachia*) (Zhou et al. 1998). We cloned any positive amplifications for both loci into the vector pGEM-T Easy according to the manufacturer's protocol (Promega). Three independent clones were sequenced for each *Wolbachia* strain to ensure that there were no polymerase errors. If more than one sequence variant was present in the sample, we sequenced a further 7 clones for a total of 10. Every clone was sequenced in both directions by an ABI automated sequencer. All sequences have been deposited in GenBank under accession numbers listed in the phylogenetic trees (Figure 1).

Multiple alignments and phylogenetic analysis.—*Wolbachia* DNA sequences from seven infected spider species were aligned together with other representative *wsp* sequences from the various supergroups, using ClustalX v. 1.8 (Thompson et al. 1997) followed by manual modifications based on the amino acid translation of different genes. We constructed phylogenetic trees using maximum likelihood (ML) and Bayesian inference (BI). ML trees were constructed with PAUP4.0 b1 (Swofford 2003) after determining the model HKY _ G with Modeltest 3.06 (Posada & Crandall 1998); BI trees were constructed using MrBayes (Huelsenbeck & Ronquist 2001) with the following parameters: brlenspr = clock:uniform, 1,000,000 generations, burnin = 1,000. Tree topologies were congruent across both phylogenetic construction methods and, therefore, only ML trees with posterior probabilities are reported.

RESULTS

We screened 930 spiders from 11 families and 31 species for *Wolbachia* infection by PCR assays with *Wolbachia*-specific *wsp* gene primers. The infection status of each tested spider species and the infection rate are listed in Table 1. We found seven spider species to be infected with *Wolbachia*. The percentage of *Wolbachia* infection among tested species was 22.6%, which is very low compared to previous surveys in insects (Jeyaprakash & Hoy 2000). There was a wide range in infection rates for the infected spider species sampled. The highest infection rate was found in *Oxyopes sertatus* L. Koch

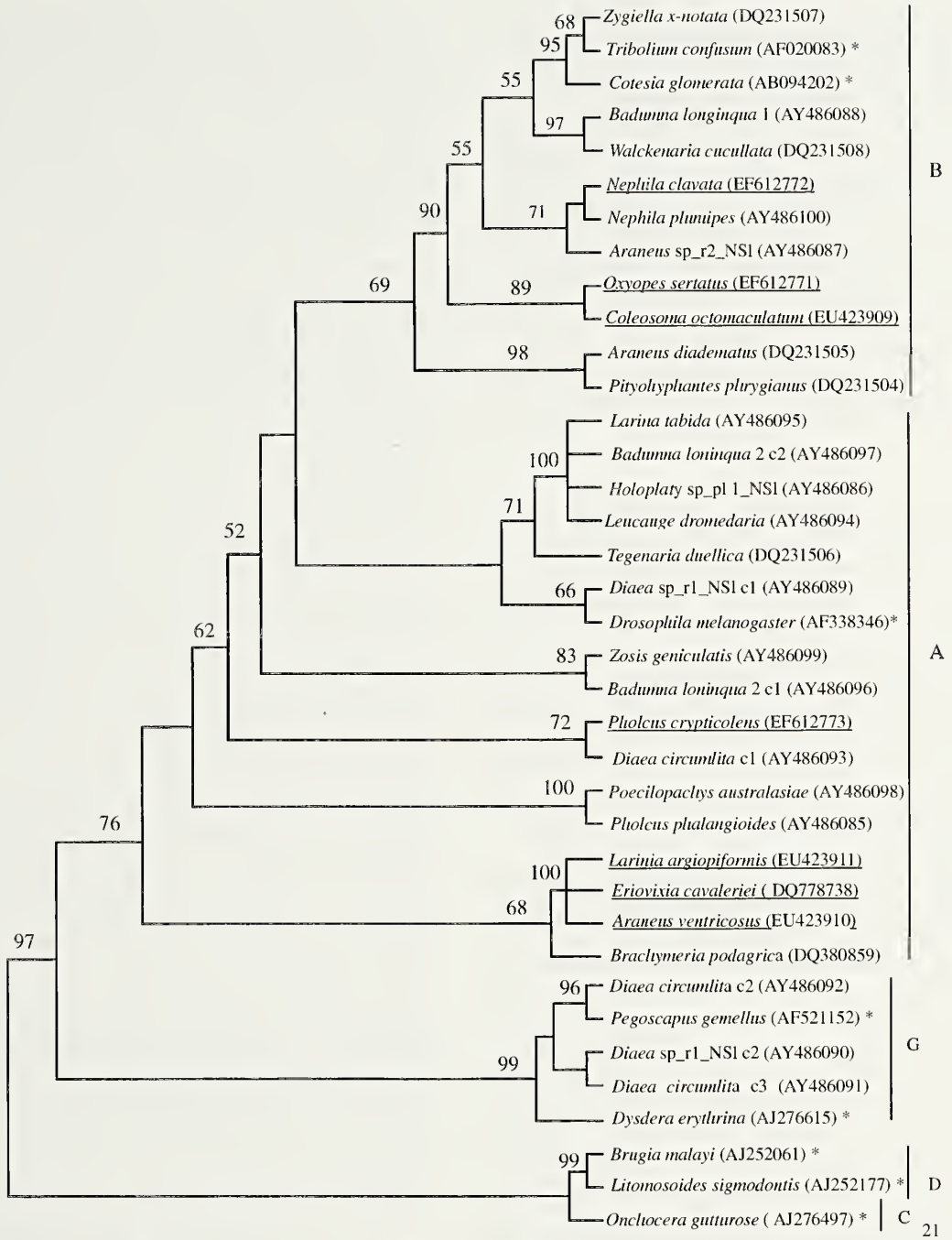

Figure 1.—Phylogeny of spider *Wolbachia* based on *wsp* gene sequence. Taxonomic names refer to host species (Table 1). Underlined designations are strains sequenced in this study. GenBank accession numbers are listed in parentheses. Asterisks after taxa indicate these strains are not from spider families. Bootstrap values of 1,000 replicates are presented above the branch (bootstrap scores less than 50 are not shown). Major supergroup lineages are indicated (A–D and G).

1877 (73.3%), and the lowest infection rate occurred in *Coleosoma octomaculatum* Bösenberg & Strand 1906 (13.3%). From the data presented here, it appears that *Wolbachia* is not widespread in the spider species that we tested.

We used the specific primers of the A and B *Wolbachia* supergroups for the *wsp* gene to identify a super-infection in the infected spiders. *Nephila clavata* (L. Koch 1878) specimens were infected with strains of both A and B *Wolbachia*; the others were infected with either strain A or B. The lengths of *wNepA* and *wNepB* (the strain A and B *wsp* genes found in *Nephila clavata*) were 522bp and 455bp, respectively. These two genes are largely different. Based on the alignment of these two genes by BLASTn in GenBank, they were only 50% similar.

Wolbachia sequences were obtained from infected individuals in each of the seven infected spider species in this study (sequences have been deposited in GenBank; accession numbers DQ778738, EF612771–612773, EU423909–EU423911). We also included a range of sequences from

closely related bacteria found in other insects, mites, and spiders (from GenBank) in our phylogenetic analyses. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). Tree topologies were congruent across both phylogenetic construction methods and therefore only ML trees with posterior probabilities are reported in Fig. 1. The *Wolbachia* phylogeny can be compared broadly with the analysis by Rowley et al. (2004), who found that *Wolbachia* strains carried by spider hosts can be from a variety of clades. The *Wolbachia* found in *Larinia argiopiformis* Bösenberg & Strand 1906, *Eriovixia cavaleriei* (Schenkel 1963), *Araneus ventricosus* (L. Koch 1878) and *Pholcus crypticolens* Bösenberg & Strand 1906 belong to the A supergroup and the other three species, *Nephila clavata*, *Oxyopes sertatus* and *Coleosoma octomaculatum* belong to the B supergroup.

DISCUSSION

We detected *Wolbachia* in 22.6% of the spider species sampled, based on the PCR assays with *Wolbachia*-specific *wsp* gene primers. That is lower than the frequency reported by Goodacre & Martin (2006), who screened 121 spider species from 9 families in southeastern Britain. They found that 37 spider species (30.6%) were infected with *Wolbachia*. Among their species, nine Araneidae were infected, but none of their twenty lycosids were infected. Similar to their results, we found that more araneids (three out of six) were infected with *Wolbachia*, whereas none of our seven lycosids were infected. The uneven distribution of *Wolbachia* among spider populations may be associated with different host (spider) conditions, host behaviors, and the efficiency of spread of *Wolbachia*.

Based on phylogenetic analysis, multiple lines of evidence indicate that at least some of the associations between spiders and *Wolbachia* are of recent origin, likely to have arisen through horizontal transmission. First, the *Wolbachia* strains found in *Larinia argiopiformis*, *Eriovixia cavaleriei*, and *Araneus ventricosus* have almost identical gene sequences and are in the same subgroup with bootstrap support of 100% for the maximum likelihood analysis. We interpreted small sequence differences among these three species to be PCR artifacts: compared to *Araneus ventricosus*, our *Larinia argiopiformis* sequence had a T instead of a C at position 118, and an A instead of a C at position 280; our *Eriovixia cavaleriei* sequence had a G instead of a T at position 518 and a T instead of an A at position 584. This finding of three distantly related Araneidae species infected with the same strain of *Wolbachia* suggests a very recent horizontal transmission. Second, closely related spider hosts do not necessarily share closely related *Wolbachia* strains. Different members of a single spider family may harbor infections from distinct supergroups (Fig. 1).

Third, using the specific primers of the A and B *Wolbachia* supergroups for the *wsp* gene, we identified one superinfection. Specimens of *Nephila clavata* were infected with distinct strains of both A and B *Wolbachia*. Spider species are generalist predators, and they prey mostly on terrestrial arthropods (insects, myriapods, and arachnids). Feeding on common prey infected with *Wolbachia* could provide the link for horizontal transfer of the same strain across related species. However, the possible role of horizontal transmission via parasitoids in spiders cannot be excluded. Spiders are known to suffer parasitoid attack from several taxa (La Salle 1990; Vavre et al. 1999). Routes and vectors of *Wolbachia* horizontal transfer remain one of the main unsolved questions. It is, for example, unclear whether horizontal transfer of *Wolbachia* is more likely to occur within the same host taxonomic group (at different levels, such as genus or species) than among different groups.

Rowley et al. (2004) reported that several *Wolbachia* infections present in the genus *Diaea* appear to be phylogenetically distinct from the A–F clades. The stability of the cluster is strengthened by the inclusion of *Wolbachia* strains infecting the spider *Dysdera erythrina* (Walckenaer 1802) and the fig wasp *Pegoscapus gemellus*. They had tentatively called this lineage supergroup G., but we did not find the G supergroup in any of the Chinese spiders tested. Thus the independence of this new supergroup should be re-examined more closely when related *wsp* sequences are published.

It has been argued that endosymbiotic bacteria that are widespread among hosts are very important in the evolution of hosts, including effects on reproduction, gene flow, and individual or population fitness, with possible impacts on reproductive isolation or extinction (Werren 1997; Werren et al. 2008). Shoemaker et al. (1999) found that behavioral isolation and *Wolbachia*-induced CI acted as complementary asymmetrical isolating mechanisms between *Drosophila recens* and *D. subquinaria*. Some unique characteristics of spider reproductive biology facilitate studies of sexual selection (Eberhard 2004), and there have already been studies examining the relationship between sexual selection in spiders and speciation (Gage et al. 2002). We suggest that, combined, comparative studies and manipulations of endosymbiont infections in spiders will help allow us to predict the relationships between bacterial infections and the evolution of host traits under sexual selection.

This study was conducted as a first step to screen the prevalence of *Wolbachia pipientis* in Chinese spiders, to determine the occurrence of super-infections in the infected spiders and the relationship of these strains to the existing *Wolbachia* supergroups. Based on these results, the effects of the bacteria upon the host, the full extent of infection throughout the order Araneae, and the practical implications of such infections all require further investigation.

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